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Patients with Chronic Granulomatous Disease Have a Reduced Peripheral Blood Memory B Cell Compartment

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In this study, we have identified an altered B cell compartment in patients with chronic granulomatous disease (CGD), a disorder of phagocyte function, characterized by pyogenic infections and granuloma formation caused by defects in NADPH activity. This is characterized by an expansion of CD5-expressing B cells, and profound reduction in B cells expressing the memory B cell marker, CD27. Both findings were independent of the age, genotype, and clinical status of the patients, and were not accompanied by altered CD5 and CD27 expression on T cells. Focusing on CD27-positive B cells, considered to be memory cells based on somatically mutated Ig genes, we found that the reduction was not caused by CD27 shedding or abnormal retention of CD27 protein inside the cell. Rather, it was determined that CD27-negative B cells were, appropriately, CD27 mRNA negative, consistent with a naive phenotype, whereas CD27-positive B cells contained abundant CD27 mRNA and displayed somatic mutations, consistent with a memory B cell phenotype. Thus, it appears that CGD is associated with a significant reduction in the peripheral blood memory B cell compartment, but that the basic processes of somatic mutation and expression of CD27 are intact. X-linked carriers of CGD revealed a significant correlation between the percentage of CD27-positive B cells and the percentage of neutrophils with normal NADPH activity, reflective of the degree of X chromosome lyonization. These results suggest a role for NADPH in the process of memory B cell formation, inviting further exploration of secondary Ab responses in CGD patients.

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Chronic granulomatous disease (CGD) is a primary immunodeficiency disorder caused by inherited defects in the NADPH oxidase complex (1–3). This enzyme complex is used by phagocytic cells to generate microbicidal superoxide and its metabolites hydrogen peroxide, hydroxyl anion, and hypochalous acid (4). As a consequence, patients are susceptible to recurrent life-threatening pyogenic infections, particularly those caused by catalase-positive bacteria and fungi. In addition, CGD patients often have poor wound healing and chronic inflammation, leading to granuloma formation. Autoimmune diseases resembling systemic lupus erythematosus, discoid lupus, pneumonitis, and inflammatory bowel disease are also experienced by patients with CGD and their relatives (4, 5). The most common form of CGD is caused by an X-linked recessive defect in gp91phox (~70% of cases), while the remainder of the cases is caused by an autosomal recessive defect in p47phox, p67phox, or p22phox (5).

Curative treatment of CGD can only be accomplished by allogeneic stem cell transplantation (aSCT), and possibly gene therapy (6, 7). As part of immune reconstitution studies in the context of nonmyeloablative aSCT for CGD, it was noted that several CGD patients displayed a substantial decrease in peripheral blood B cells expressing CD27 before undergoing aSCT (8). CD27 is considered an immunophenotypic marker identifying peripheral blood memory B cells (9, 10). This is based upon morphology, Ig production, and the presence of somatically mutated VH region genes, encoding the H chains of Ig molecules on B cells that express CD27. In naive, CD27-negative, B cells, these genes are found to be mostly in a germline configuration (9, 10). Clinical correlation has been demonstrated by the finding of greatly reduced CD27-positive B cells in patients with primary immunodeficiency disorders that affect B cell function and memory B cell formation (11–13).

The finding of reduced CD27-positive B cells in CGD patients may be linked to the observation that B cells possess a superoxide generating system with structural homology to the NADPH oxidase system of phagocytes, but with 50- to 100-fold less oxidase activity (14). A role for the NADPH oxidase system in B cells is suggested by oxidase activity in response to surface Ig cross-linking and the prevention of proliferation of human peripheral blood B cells by interference with oxidase activity (15, 16). This current study was undertaken to characterize the decrease in CD27-positive B cells in patients with CGD and determine whether this is linked to their defects in NADPH oxidase. The results reveal a profound reduction in the contribution of CD27+ B cells to the peripheral B cell compartment in patients with CGD, and this appears to correlate with the defective NADPH oxidase system.
Materials and Methods

Study subjects

The patient population consisted of 50 patients with CGD (CGD group). Most patients were receiving prophylactic antimicrobials, and several patients were receiving s.c. IFN-γ therapy. In addition, 21 carriers of patients with the X-linked form of CGD were studied, as well as several heterozygous parents of autosomal recessive CGD patients. Participating patients and their family members provided written consent for evaluation and follow-up at the National Institutes of Health. A control group, consisting of 30 healthy, nonsmoking, adult volunteers (healthy control (HC) group), provided reference ranges for lymphocyte markers and soluble CD27 (sCD27) levels. In addition, healthy volunteers were used as controls in somatic mutation, CD27 mRNA, and dihydrothoradamine (DHR) experiments. All experiments were reviewed and approved by the appropriate institutional review board.

Flow cytometry

Peripheral blood specimens were obtained by phlebotomy on site or were mailed in and studied the next day. There were no differences in any lymphocyte marker between freshly obtained or transported specimens (data not shown). Anticoagulated (EDTA) samples were stained using the whole blood lysis method and analyzed on a dual-laser FACSCaliber (BD Biosciences) using CellQuest software (BD Biosciences), previously described (13, 17). Control samples from healthy volunteers were analyzed contemporaneously with experimental samples. In all experiments, B cell subsets were identified by expression of either CD19 or CD20. Other Abs included: anti-CD5, anti-CD21, anti-CD22, anti-CD23, anti-CD25, anti-CD38, anti-CD40, anti-CD45R0, anti-CD62L, anti-CD80, and anti-CD86. Memory B cells were immunophenotypically identified by CD27 in combination with goat F(ab′)2 anti-human IgD and goat F(ab′)2 anti-human IgM polyclonal Abs. T cells and T cell subsets were identified with the directly conjugated mouse anti-human mAbs: anti-CD3, anti-CD5, anti-CD27, and anti-CD70. NK cells by a combination of anti-CD16 and anti-CD56, evaluated on CD3-negative lymphocytes (quality control purpose). Irrelevant, directly conjugated, mouse anti-human mAbs of the IgG1, IgG2a, and IgG2b subclasses were used to define background staining. All mAbs were obtained from BD Biosciences and Beckman Coulter, and used as recommended by the manufacturer. Lymphocytes were identified by forward and side scatter, and the lymphocyte gate was checked using the CD45/CD14 LeucogATE reagent (BD Biosciences). To calculate the absolute numbers of each lymphocyte subset, the percentage of positive cells was multiplied by the absolute PBL count. For intracellular detection of certain lymphocyte markers, pretreatment using FIX & PERM (Caltag Laboratories) was performed, according to the manufacturer’s instructions. Anti-CD79a, an intracellular B cell-specific marker, was used as a control for the permeabilization procedure.

sCD27 ELISA

sCD27 in plasma or serum were determined by the PeliKine compact human sCD27 ELISA kit (CLB), according to the manufacturer’s instructions. Briefly, an anti-CD27 mAb was coated to polystyrene microtiter wells, which binds sCD27 present in serum/plasma and standard. After washing, a biotinylated second anti-CD27 mAb was added, forming a sandwich with plate-bound anti-CD27 and sCD27, followed by washing, addition of HRP-conjugated streptavidin, washing, and addition of substrate solution for enzymatic color development. Absorbance of patient samples and standards was measured at 450 nm in an ELISA reader. The concentration of sCD27 in serum/plasma was determined by interpolation with the standard curve, generated from seven sCD27 standards, ranging from 1.56 to 100 U/ml. A reference range was established from 30 healthy volunteers, analyzed concurrently with patient samples (17).

DHR fluorescence assay of granulocytes

DHR assays of granulocytes were performed, as published (18, 19). Briefly, after red cell lysis, leukocytes were loaded with 1.8 μl of 2 mM DHR for 5 min at 37°C in the presence of 1000 U/ml catalase. After DHR loading, cells were left unstimulated or were stimulated with PMA at a concentration of 1.56 to 100 U/ml. A reference range was established from 30 healthy volunteers and sCD27 standards, ranging from 1.56 to 100 U/ml. A reference range was established from 30 healthy volunteers, analyzed concurrently with patient samples. For patients and HC, a stimulation index was generated by dividing the geometric mean channel FL2 fluorescence of the stimulated granulocytes by the geometric mean channel FL2 fluorescence of the unstimulated granulocytes. A defective DHR assay was defined by an stimulation index of <115, previously determined by the 95% confidence interval (CI) of 40 healthy adult volunteers (18, 19).

Quantitative CD27 RT-PCR

RNA from IgD+/-CD27+ B cells, IgD-/-CD27+ B cells, or total B cells isolated from CGD, and control samples was prepared using TRIzol (Invitrogen Life Technologies) and RNeasy columns (Qiagen), according to the manufacturer’s instructions. Fifty nanograms of RNA was reverse transcribed and amplified in triplicate using the Superscript One-Step RT-PCR kit (Invitrogen Life Technologies), according to the manufacturer’s instructions, with the addition of a 1/50 dilution of ROX reference dye (Invitrogen Life Technologies). Exon-spanning primer and probe sets for human CD27 were obtained from Applied Biosystems (Assays On-Demand). Primer efficiency was measured by serial dilution of template and was used to calculate the final relative abundance of CD27 mRNA for each B cell population.

Somatic mutation analysis

PBMC from three CGD patients and two age-matched HC were isolated using a Ficoll gradient (Ficoll-Paque) and stained with the mAbs: FITC-labeled anti-human IgD, PE-labeled anti-human CD27, and allophycocyanin-labeled or PE-labeled anti-human CD19 (BD Biosciences). The CD19+ B cells, the CD19+IgD+/–CD27+ B cells, and the CD19+IgD-/CD27+ B cells were sorted using a DakoCytomation MoFlo (DakoCyto- mation) into 5-nl tubes containing 500 μl of 1× PBS. The total sorted cells were diluted to a final concentration of 1–5 cells per 5 μl of PBS, and then aliquoted into 96-well PCR plates containing 10 μl of lysis buffer (2× PCR buffer, 6.4 ng/ml protease K (Sigma-Aldrich). Cells were lysed for 60 min at 56°C, followed by a denaturation step of 95°C for 10 min to isolate genomic DNA. The total genomic DNA was amplified with an initial untempered primer extension preamplification PCR, after which individual VH genes were amplified, sequenced, and analyzed, as described previously (20, 21). The maximal error rate of the amplification and sequencing technique has been documented to be 1 × 10−5 (22). Thus, few, if any, of the nucleotide changes encountered in this analysis can be ascribed to PCR amplification and sequencing error.

A total of 51 VH3 sequences from CGD CD19+ B cells, including 10 nonproductive and 41 productive rearrangements, together with Vh3 and Vh4 rearrangements from control CD19+IgD+/–CD27+ B cells (n = 49), CGD CD19+IgD+/–CD27+ B cells (n = 19), and CGD CD19+IgD-/CD27+ B cells (n = 8), were analyzed. All rearrangements were matched to their closest germline counterparts using the web-based program JOIN- SOLVER (21). If mismatches were found in the V gene segment (up to aa 92), the sequence was regarded as mutated (22). The frequency of VH mutations (percent) was determined by dividing the total number of VH mutations by the total number of VH bases and multiplying by 100.

Statistical analysis

Quantitative data were expressed as medians and percentiles. Results obtained by flow cytometry and sCD27 ELISA were compared by Mann-Whitney U test. Data regarding CD27 RT-PCR and somatic mutation analysis were compared by Student’s t test. The Spearman rank correlation coefficient was used to calculate quantitative correlations. All p values were two sided and regarded statistically significant if p < 0.05.

Results

Patient characteristics

The CGD group consisted of 37 patients (74%) with the X-linked form and 13 patients (26%) with the autosomal recessive form of CGD. The mean age of the group was 16 years, with a range of 2–62 years. The majority of CGD patients were clinically stable on daily prophylactic antimicrobial therapy. A subgroup of patients also received prophylactic s.c. IFN-γ therapy. Their results were comparable to the other patients. The X carrier group consisted of 16 mothers, three sisters, and one grandmother of X-linked CGD patients. With the exception of one, X-linked carriers were healthy (23). Several (healthy) heterozygous parents of patients with the autosomal recessive form of CGD were also studied.

Immunophenotype of B cells in CGD patients

CGD patients displayed a significant B cell lymphocytosis, involving a subset of B cells, defined by CD5 expression. A median of...
60% (90% CI 27–80%) of B cells was CD5 positive, compared with a median of 39% (90% CI: 25–56%) in HC (Fig. 1A). The expansion of CD5-positive B cells did not significantly change when analysis was confined to patients older than 16 years of age, to rule out age dependency of this finding.

In addition to the expansion of CD5-positive B cells, the CGD group showed a highly significant reduction in the percentage and absolute number of peripheral blood B cells that expressed CD27 (Fig. 1B). An example, showing both CD5 and CD27 expression on CD19-positive B cells from a representative CGD patient and an HC, is depicted in Fig. 1C. In the CGD group, a median of 3.3% of B cells was CD27 positive, compared with 31.9% in the HC group. This corresponded to a median of 10 CD27-positive B cells per μl in the CGD group vs a median of 50 CD27-positive B cells per μl in the HC group. As can be seen in Fig. 1B, the percentage of B cells that expressed CD27 was quite variable in the HC group.

FIGURE 1. CD5 and CD27 expression in CGD patients. A, Boxplot depicting the 10th, 25th, 50th (median), 75th, and 90th percentiles of the percentage of CD5+B cells in 50 CGD patients and 30 HC. B, Boxplots depicting the 10th, 25th, 50th (median), 75th, and 90th percentiles of the percentage (left boxplot) and absolute number (right boxplot) of CD27+B cells in the GCD group and HC group. C, Dot plots of CD5 and CD27 expression on gated CD19+ lymphocytes from an HC (left) and a representative X-linked CGD patient (right). Numbers denote the percentage of B cells that express CD5 and CD27, respectively, and are based upon isotype control staining and verified using both a CD5/CD27-negative control population in the lower left quadrants (NK cells) and a CD5/CD27-positive control population in the upper left quadrants (T cells). D, Panel of dot plots showing lack of up-regulation of B cell activation markers (CD25, CD45RO, and CD80) or down-regulation of B cell markers (CD40, CD62L) in a representative CGD patient, compared with an HC. All events are gated on B cells (identified by CD20 expression).
CD27-positive B cells, or absence of CD27. Two main causes were considered: absent or altered CD27 expression on B cells, or absence of CD27+ B cells. The first approach taken was based on the observation that in immune-mediated disorders and malignancies, CD27 is proteolytically cleaved from the surface of cells, a process common to members of the TNF superfamily (17, 25, 26). This possibility was evaluated by measuring sCD27 levels in plasma or serum of 25 CGD patients, and comparing these with the levels found in a reference group of 30 HC.

As can be seen in Fig. 2, there was no significant increase in sCD27 in the CGD group, as compared with the HC group. The wider distribution of sCD27 data in the CGD group was largely the result of the inclusion of several patients with ongoing clinically relevant infections. T cells from CGD patients did not express increased levels of CD70, the ligand of CD27, suggesting no increased activity of the CD27/CD70 receptor/ligand pair and associated metalloproteinase-induced cleavage (data not shown). The absence of elevated sCD27 was consistent with the lack of intracellular CD27 protein staining by flow cytometry, observed in B cells evaluated from three representative patients (data not shown).

The results established that cleavage of surface CD27 is not up-regulated and CD27 protein is not retained in the cytoplasm of the expanded population of CD27-negative B cells.

The next experiments examined CD27 mRNA levels to confirm that CD27-negative B cells were indeed naive (9, 10). These studies consisted of evaluating quantitative CD27 mRNA levels in separated naive (IgD+/CD27−) and memory (IgD−/CD27+) B cell populations, from three representative CGD patients, and naive (IgD+/CD27+) and total B cell populations from two age-matched HC (all identified by CD19 expression). As presented in Fig. 3A, the relative amount of CD27 mRNA in the naive B cell population from CGD patients was very low, and similar to the findings in the control naive B cell population. In contrast, both the memory B cell population from CGD patients and total B cells from age-matched controls showed significantly higher expression of CD27 mRNA ($p < 0.05$).

To establish that these sorted populations indeed represented naive and memory B cells, somatic mutation analysis was performed. As is summarized in Fig. 3B, the results revealed minimal evidence of somatic mutation of the Ig H chain V region genes in the IgD+/CD27− B cell populations from either CGD patients or controls.

### FIGURE 2.

sCD27 levels. Boxplots depicting the 10th, 25th, 50th (median), 75th, and 90th percentiles of sCD27 levels as detected in plasma/serum by CD27 ELISA kit in 30 HC and 25 CGD patients.

### FIGURE 3.

A. The relative amount of CD27 mRNA was measured in pooled naive (CD19+/IgD+/CD27−) and memory (CD19+/IgD−/CD27+) B cells from CGD samples and compared with pooled whole B cells (CD19+) and naive B cells (CD19+/IgD+/CD27−) from control samples, to assess whether CD27-negative B cells had previously expressed CD27. B. The frequency of somatic mutations in the $V_H$ region genes was calculated in naive (CD19+/IgD+/CD27−) and memory (CD19+/IgD−/CD27+) B cells from CGD samples and compared with the frequency in control naive B cells, to determine whether the CGD CD19+/IgD−/CD27− B cell population was indeed naive (unmutated).
control subjects (0.3 ± 0.44 and 0.4 ± 1.2%, respectively). Moreover, the somatic mutation frequency was lower than in the IgD-/CD27− (memory) B cell population, confirming that the IgD+/CD27+ B cell population from CGD patients is in a naive state, similar to controls (3.9 ± 4.7 and 7.4 ± 3.8%, respectively). Additionally, in the CDRs, the replacement to silent ratio (R/S ratio) of mutations in the CGD IgD+/CD27− (memory) B cells was comparable to the control memory population (3.1 and 4.0, respectively). Similarly, the R/S ratios in the framework regions of the CGD IgD+/CD27− (memory) B cells were comparable to the control memory population (0.8 and 1.4, respectively). These values are within the reported range for CDR and framework region R/S ratios of normal human peripheral B cells (27).

Thus, from these results, it was concluded that CD27 mRNA was appropriately absent from CD27-negative B cells confirmed to be naive B cells by somatic mutation analysis, and conversely that CD27 mRNA was appropriately present in the memory B cell population in CGD.

To further explore the process of somatic mutation and memory B cell formation in CGD patients, additional studies of somatic mutation of Ig genes were undertaken in the B cells from CGD patients. This analysis was restricted to the VH3 family of H chain genes, which is the largest VH family and the one used most frequently (27). A total of 41 productive rearrangement and 10 non-productive rearrangements was analyzed. Within the productively rearranged VH3DJH rearrangements, 34 of 41 (83%) contained mutations, whereas 9 of 10 nonproductive rearrangements contained mutations. There were 108 mutations in the 7679 bp of the productive rearrangements for an overall mutational frequency of 1.4 × 10−2. In the nonproductive repertoire, the mutational frequency was 1.2 × 10−2 (29 mutations in 2353 bp). Although the frequency of mutations was somewhat less than that found in normal adults, it was significantly greater than the PCR error rate (1 × 10−4).

Together with the previous results, it appears that there are no fundamental differences in the V(D)J rearrangement and selection mechanisms in patients with CGD, reflective of memory B cell generation, but that the memory B cell pool in peripheral blood is significantly smaller as compared with HC.

**X-linked carriers reveal a link between NADPH oxidase activity and B cell phenotype**

It was noted that the distribution of CD27-positive B cells was highly variable among several carriers of X-linked CGD, even more so than in the HC group. In addition, it has been appreciated previously that the ratio between granulocytes with normal NADPH oxidase activity and granulocytes with defective activity, reflective of the normal vs the abnormal gp91phox alleles on the X chromosomes, varies greatly (4). An illustrative case is shown in Fig. 4A, demonstrating the DHR 123 assay of oxidase activity of granulocytes from the mother and grandmother of an X-linked CGD patient, sharing the same genotype. The assay revealed the typical presence of two populations of granulocytes, corresponding to the normal and abnormal gp91phox alleles. However, the distributions of granulocytes with normal vs abnormal NADPH oxidase activity were strikingly divergent, with 85 and 22% abnormal granulocytes, and 15 and 78% normal granulocytes, respectively. For comparison, DHR assay results from an HC, and the affected son/grandson of these two carriers are shown in Fig. 4B. In the two X-linked carriers, CD27 expression on B cells was determined in parallel with the DHR assay. The results showed that the percentage of CD27-positive B cells appeared to correlate with the percentage of granulocytes with normal oxidase activity (Fig. 4B).

To analyze this relationship in greater detail, DHR assays were performed in a total of 21 carriers, in parallel with B cell immunophenotyping. The results demonstrated a highly significant correlation (Spearman’s ρ = 0.8, p = 0.001) between the percentage of DHR-positive granulocytes (representing the normal gp91phox allele) and the percentage of CD27-positive B cells (Fig. 4C). Thus, this analysis reveals a significant correlation between a measurement in granulocytes and a measurement in B cells. To determine whether this correlation affected isotype-switched (IgM/IgD−) and nonisotype-switched (IgM+/IgD+) CD27+ B cell subsets to the same degree, DHR-positive granulocytes were correlated with these subsets in 18 X carriers (in which IgM and IgD expression were available). As is shown in Fig. 4C, the correlation is stronger for isotype-switched than for nonisotype-switched CD27+ B cells. There was also a significant inverse relationship (ρ = −0.5, p = 0.03) between the percentage of DHR-positive granulocytes and the percentage of CD5-positive B cells, which suggested an alteration of the B cell compartment in CGD patients (data not shown).

In HC, the distribution of total CD27-positive B cells into isotype-switched and nonisotype-switched subsets generally favors the latter. This distribution could not be reliably analyzed in the CGD group due to the profound reduction in CD27+ B cells. Given the DHR/memory B cell subset correlations depicted in Fig. 4C, the question was asked whether this distribution was different in the X carriers. The results, as shown in Fig. 4D, revealed that this was indeed the case. When compared with the HC group, the distribution was different, showing that the isotype-switched CD27+ B cell subset was comparable to this subset in HC, whereas for the nonisotype-switched subset, the discrepancy between X carriers and HC was more pronounced and statistically significant.

Thus, both immunophenotypic B cell abnormalities observed in CGD patients were observed in X-linked carriers, proportional to the distribution of cells with an intact vs a mutant NADPH oxidase system. Moreover, there appeared to be a differential effect of the NADPH oxidase system on switched vs nonswitched memory B cells. Heterozygous parents of autosomal recessive CGD patients have one single population of normal granulocytes, and likewise have normal CD27 expression on their B cells (data not shown).

**Discussion**

The key finding of this study is that patients with CGD have an altered peripheral B cell compartment, characterized by two findings: a significantly increased population of CD5-positive B cells and a significantly decreased population of CD27-positive B cells.

The expansion of CD5-positive B cells was observed regardless of age, genotype, clinical condition, or specific CGD-related manifestations. The origin and relevance of CD5-positive B cells in humans remain somewhat elusive, and most likely reflect a pleiotropic role for CD5 in different B cell populations and different situations (28, 29). The origin or significance of our observation remains unclear at this point; the finding of a significant inverse relationship between the percentage of CD5-positive B cells and the percentage of granulocytes with intact oxidase activity in healthy (adult) X-linked carriers would suggest that the NADPH oxidase system of B cells is somehow involved. The well-recognized link between CD5-expressing B cells and autoimmunity could be of relevance given the increased frequency of discoid and systemic forms of lupus in patients with CGD and X-linked carriers (4, 5, 30–32).

A profound reduction in B cells expressing CD27 was found in patients with CGD, regardless of genotype, age, or clinical condition. Virtually all patients displayed a level of CD27-positive B
FIGURE 4. Link between CD27 expression and the NADPH oxidase system. A, DHR fluorescence assays were performed in two related carriers of X-linked CGD concomitantly with B cell immunophenotyping. The histograms show the percentages of granulocytes with intact (15 and 78%, respectively) and defective NADPH oxidase activity in the two X carriers in response to PMA stimulation, while the corresponding dot plots reveal the percentage of B cells that express CD27 (5.9 and 37.5%, respectively). Dashed lines in the histograms represent the results from the unstimulated control specimens run concurrently. B, Histograms representing DHR assays in HC and X-linked CGD patient. Dashed lines in the histograms represent the results from the unstimulated control specimens. C, Results of simultaneous DHR assays and CD27 phenotyping in X carriers were analyzed with the Spearman rank correlation coefficient between the population of granulocytes with intact NADPH oxidase activity (DHR+) and the percentage of total CD27+ B cells (left) and isotype-switched (ISO, upper right) and nonisotype-switched (NON-ISO, lower right) CD27+ B cells. Note: best fit line extending beyond data points represents hypothetical interpretation. D, Boxplots depicting the 10th, 25th, 50th (median), 75th, and 90th percentiles of the percentage of total CD27+ B cells, nonisotype-switched (NON-ISO), and isotype-switched (ISO) CD27+ B cells, respectively, in X carriers (XC) and HC. Comparisons by Mann-Whitney U test.
cells that fell below our established reference range, obtained from HC subjects. In addition, as can be seen in Fig. 1A, the distribution of CD27 data was quite narrow, suggestive of a closely shared mechanism, despite the differences in age, genotype, or clinical condition. The question was asked whether decreased CD27 expression resulted from a specific lack of CD27 expression on B cells, or a decreased generation of CD27-expressing (memory) B cells. Starting with the first possibility, we did not find significantly increased levels of sCD27, consistent with proteolytic cleavage, among the CGD patients, nor did we find intracellular retention of CD27 protein (17, 25, 26). Next, it was determined whether CD27-negative B cells had previously expressed CD27, as a consequence of differentiation into memory B cells, but that continued expression of this marker was negatively influenced by the defective NADPH oxidase system in CGD B cells. The approach was to measure CD27 mRNA levels in CD27-negative and CD27-positive B cell populations. This was combined with somatic mutation analysis of sorted B cell subpopulations, to identify these two B cell subpopulations in terms of naive vs memory status. As shown in Fig. 3A, in both patients with CGD and age-matched controls, CD27 mRNA was appropriately absent from CD27-negative B cells that by somatic mutation analysis were deemed to be naive B cells, whereas CD27-positive B cells contained CD27 mRNA, and had the memory B cell configuration of the Ig H chain genes. Our studies confirmed that there are no major differences in the somatic mutation processes in B cells from patients with CGD compared with controls, indicating that those B cells that participate in the germinal center reaction do so in an apparently normal fashion. Moreover, the somatically mutated memory B cells that emerge from the germinal center appear to have normal transcription and translation of CD27, as well as control of expression, and do not demonstrate excessive loss of this surface protein.

We hypothesize that the profound and consistent reduction in CD27-expressing B cells, regardless of age, clinical condition, or genotype of CGD patients, is linked to the defective NADPH oxidase complex or components thereof. This is substantiated by the findings in the X-linked carriers, demonstrating a highly significant correlation between the percentage of CD27-positive B cells and the percentage of granulocytes with normal NADPH oxidase activity. Interestingly, this affects nonisotype-switched memory B cells more than isotype switched, although the basis for this remains to be defined. The CD27 findings in CGD patients are comparable to CD27 results commonly observed in primary immunodeficiency disorders, associated with a defective germinal center reaction (X-linked hyper-IgM syndrome being the prototypic disorder) (11, 13). As there are no obvious indications that humoral immunity is similarly adversely affected in CGD (serum IgG, IgA, and IgM levels were obtained in 40 of 50 subjects and were found to be essentially within normal limits), it is difficult to envision that CDG and disorders, such as X-linked hyper-IgM syndrome, share a similar pathogenic mechanism. In this regard, in a recent comprehensive review of histopathological findings in CGD, no absent or grossly abnormal germinal centers were reported, while one lymph node from a 5-year-old CGD patient was read as normal (33).

Previously, it has been shown that there is a connection in B cells between the production of reactive oxygen species (ROS) via the NADPH oxidase complex and the process of tyrosine phosphorylation and, more specifically, between ROS production and CD40 ligation that involves NF-kB activation (16, 34). Further evidence of the relationship between NADPH oxidase and CD40 was recently provided by studies, showing that ROS production by the NADPH oxidase system was induced by CD40 engagement via the pathway that involves TNFR-associated factor 3 association with components of the NADPH oxidase complex (35). Thus, signaling through the CD40-CD40 ligand system, a crucial element in the germinal center reaction and generation of memory B cells, may be adversely affected in patients with CGD and could explain the contracted compartment of memory B cells in peripheral blood (36). The lack of impact by the clinical phenotype of the patients on the distribution of CD27-positive B cells and the observations made in the healthy X-linked carriers seem to dispel other explanations, including decreased recruitment of naive B cells into the germinal centers, related to an altered anatomical integrity of lymphoid tissue and/or interruption of memory B cell expansion in favor of differentiation into plasma cells due to an altered chemokine and/or cytokine environment (37, 38).

In summary, patients with CGD have an altered B cell compartment, characterized by an expansion of CD5-positive B cells, and profound reduction of CD27-positive, memory, B cells, but with an apparently intact somatic mutation process. Studies in CGD patients and X-linked carriers link this observation to dysfunction of the NADPH oxidase system. It remains to be determined how this system is involved in the germinal center reaction and memory B cell formation, and if/how this relates to B cell function and/or homeostasis in patients with CGD.

Disclosures
The authors have no financial conflict of interest.

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